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Free fatty acid receptor 1 (FFAR1/GPR40) signaling affects insulin secretion by enhancing mitochondrial respiration during palmitate exposure

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ABSTRACT

Fatty acids affect insulin secretion via metabolism and FFAR1-mediated signaling. Recent reports indicate that these two pathways act synergistically. Still it remains unclear how they interrelate. Taking into account the key role of mitochondria in insulin secretion, we attempted to dissect the metabolic and FFAR1-mediated effects of fatty acids on mitochondrial function. One-hour culture of MIN6 cells with palmitate significantly enhanced mitochondrial respiration. Antagonism or silencing of FFAR1 prevented the palmitate-induced rise in respiration. On the other hand, in the absence of extracellular palmitate FFAR1 agonists caused a modest increase in respiration. Using an agonist of the M3 muscarinic acetylcholine receptor and PKC inhibitor we found that in the presence of the fatty acid mitochondrial respiration is regulated via $G\alpha_q$ protein-coupled receptor signaling. The increase in respiration in palmitate-treated cells was largely due to increased glucose utilization and oxidation. However, glucose utilization was not dependent on FFAR1 signaling. Collectively, these results indicate that mitochondrial respiration in palmitate-treated cells is enhanced via combined action of intracellular metabolism of the fatty acid and the $G\alpha_q$-coupled FFAR1 signaling. Long-term palmitate exposure reduced ATP-coupling efficiency of mitochondria and deteriorated insulin secretion. The presence of the FFAR1 antagonist during culture did not improve ATP-coupling efficiency, however, it resulted in enhanced mitochondrial respiration and improved insulin secretion after culture. Taken together, our study demonstrates that during palmitate exposure, integrated actions of fatty acid metabolism and fatty acid-induced FFAR1 signaling on mitochondrial respiration underlie the synergistic action of the two pathways on insulin secretion.

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1. Introduction

Free fatty acids (FFAs) play an essential role in the regulation of insulin secretion. At low glucose levels, FFAs are used as a substrate for generation of ATP and maintain insulin secretion [1]. At high glucose conditions, β-oxidation is inhibited by a product of the glycolytic pathway, malonyl-CoA, and fatty acids are directed towards formation of triacylglycerol (TAG) [2,3]. The anabolic and catabolic reactions between long-chain acyl Co-As (LC-CoA) and TAG, known as glycerolipid/free fatty acid (GL/FFA) cycle, produce lipid signaling molecules including LC-CoAs, phosphatic acids, monoacylglycerol and diacylglycerol (DAG), all of which stimulate insulin secretion [4]. In addition to its role as a nutrient, FFAs serve as ligands and influence insulin secretion by interacting with G-protein coupled receptors (GPCRs) on the plasma membrane [5,6]. One of the GPCRs that is highly expressed in beta cells is the free fatty acid receptor 1 (FFAR1 or GPR40) [5,6]. Activation of the receptor leads to activation of phospholipase C (PLC) and hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) into DAG and inositol triphosphate (IP_3). DAG and IP_3 potentiate insulin secretion by activating protein kinase C (PKC) and triggering ER Ca^{2+} release, respectively [7,8]. Recently, FFAR1 agonists have been developed as potential therapeutic agents for the treatment of type 2 diabetes [9–12].

In contrast to short-term effects, long-term exposure of beta cells to FFAs impairs insulin secretion and triggers apoptosis [13]. The deleterious effects of FFAs have been linked to altered glucose/fatty acid oxidation cycle [13], decreased NADPH content [14], endoplasmic reticulum (ER) stress [15] and partitioning towards formation of toxic ceramide species [16]. Also, FFAR1 signaling has been implicated in the long-term deleterious effects of FFAs [17–20].

We have recently demonstrated that fatty acid metabolism and FFAR1 signaling act synergistically on insulin secretion [17]. Reduced β-oxidation of fatty acids in the presence of a FFAR1 antagonist pointed out mitochondria as a site where the two pathways may converge [17].

Abbreviations: 2-DG, 2-deoxyglucose; BSA, bovine serum albumin; DAG, diacylglycerol; FBS, fetal bovine serum; FFA, free fatty acid; FFAR1, free fatty acid receptor 1; GL/FFA, glycerolipid/free fatty acid; GPCR, G-protein coupled receptor; GSIS, glucose-stimulated insulin secretion; IP_3, inositol triphosphate; LC-CoA, long-chain acyl CoAs; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; OCR, oxygen consumption rate; CPT1, carnitine palmitoyltransferase 1; TAG, triacylglycerol.

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It is known that this organelle is pivotal in beta-cell function. Uncoupling of respiration from ATP synthesis is essential for the regulation of ATP/ADP ratio and insulin secretion [21]; and beta cells depleted of mitochondria are unable to properly change insulin secretion in response to metabolic changes [22,23]. Taking into account the aforementioned, we decided to investigate the effects of fatty acid metabolism and FFAR1 signaling on mitochondrial function.

2. Materials and methods

2.1. Culture of cells and human islets

Mouse insulinoma MIN6 cells (a kind gift from Prof. Jun-Ichi Miyazaki, Osaka University, Japan) and human HEK293 cells were cultured in Dulbecco's Modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 25 mM glucose and supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 55 μM β-mercaptoethanol at 37 °C and 5% CO_2. Experiments on MIN6 cells were performed between passages 21–30.

Human islets were obtained from brain-dead otherwise healthy individuals from the Islet Transplantation Unit at Uppsala University. Islets were cultured in CMRL 1066 medium (Invitrogen) containing 5.5 mM glucose and supplemented with 10% FBS. Ethical permission to use human islets was obtained from the Regional Ethical Review Board in Uppsala (EPN number 2010/006; 2010-02-10).

2.2. Free fatty acid preparation

Palmitate (Sigma Aldrich, St. Louis, MO, USA) was prepared as 100 mM stock solution dissolved in 50% ethanol. Stock solution was diluted in culture medium to 0.5 mM concentration and allowed to complex for 30 min at 37 °C with fatty acid free bovine serum albumin (BSA) (Boehringer Mannheim GmbH, Mannheim, Germany) to a final molar ratio of 6.6 to 1 [17].

2.3. Short- and long-term treatment of cells and human islets

Cells/islets were treated with 0.5 mM palmitate (Sigma Aldrich) in the absence or presence of FFAR1 antagonists; 2 μM ANT203, 2 μM ANT825 (compound 39 in [24]) (both compounds from AstraZeneca, Macclesfield, UK) or 10 μM DC260126 (Tocris Bioscience, Bristol, UK).

Short-term treatment was performed for 1 h in XF assay medium (Seahorse Biosciences, North Billerica, MA, USA) set to pH 7.4 and supplemented with 25 mM glucose. Insulin secretion and oxygen consumption rate (OCR) were measured during culture.

Long-term treatment was performed for 48 h in complete DMEM culture medium. Glucose-stimulated insulin secretion (GSIS) and OCR were determined after treatment.

2.4. Down-regulation of FFAR1 by short hairpin RNA

FFAR1 was down-regulated by using the short hairpin RNA (shRNA) 5′-CCGGGCCCGTCTCAGTTTCTCCATTCTCGAGAATGGAGAAACTGAGACGG GCTTTTT-3′ (Sigma Aldrich). pLKO.1-puro non-Mammalian shRNA control plasmid DNA (Sigma Aldrich) was used as a negative control. Transfection was performed in 96-well plates by adding 50,000 cells to a mixture containing 1 μl Lipofectamine 2000 (Invitrogen) and 0.3 μg DNA in 50 μl OptiMEM (Invitrogen). After overnight incubation, the transfection medium was replaced with the culture medium for another 72 h.

2.5. Measurement of FFAR1 mRNA level by real-time PCR

Total mRNA was isolated from MIN6 cells using NucleoSpin® RNA (Macherey-Nagel, Duren, Germany) and reversely transcribed into cDNA with SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The real-time PCR was performed in 10 μl volume using Dynamo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland). The following primers were used for amplification: FFAR1 (forward primer, 5′-CCATTCTGCTCTTCTTTCTG-3′ and reverse primer, 5′-GGGT TTATGAAACTAGCCAC-3′), β-actin (forward primer, 5′-TCTGTGTGGA TTGGTGGCTC-3′ and reverse primer, 5′-GACTCATCGTACTCCTGCTT GCT-3′). FFAR1 mRNA level was normalized to the housekeeping gene β-actin using the following formula: target amount $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [C_t\ (\text{GPR40 KO}) - C_t\ (\beta\text{-actin KO})] - [C_t\ (\text{GPR40 control}) - C_t\ (\beta\text{-actin control})]$ [25].

2.6. Oxygen consumption measurements

Mitochondrial respiration was determined by measuring OCR in the Extracellular Flux Analyzer XF96e (Seahorse Biosciences). Assays were performed in XF assay medium (Seahorse Biosciences) set to pH 7.4 and supplemented with 25 mM glucose.

Mitochondrial function was determined by measuring basal respiration, ATP-coupled respiration, proton leak and maximal respiratory capacity. Basal OCR was measured during the last 30 min of 1-h culture. All measurements were corrected for non-mitochondrial OCR, which was measured by adding inhibitors of electron transport chain; rotenone (5 μM) and antimycin (5 μM). Mitochondrial OCR was estimated by subtracting OCR measurement after rotenone/antimycin addition from OCR measurement before oligomycin addition. ATP-coupled respiration was assessed by the addition of ATP synthase inhibitor oligomycin (4 μM). OCR measurement after oligomycin addition was subtracted from OCR measurement before oligomycin addition. The drop in OCR induced by oligomycin addition reflects ATP-coupled respiration. Proton leak OCR was estimated by subtracting OCR measurement after addition of rotenone/antimycin from OCR measurement after addition of oligomycin. Maximal respiratory capacity was determined by adding 4 μM ionophore FCCP.

OCR was, in addition, measured in the presence of FFAR1 agonists TUG-499 (2 μM) (Merck Millipore, Darmstadt, Germany) and AS2034178 (2 μM) (Tocris Bioscience). Also, OCR was measured in the presence of 2-deoxyglucose (2-DG) (100 mM), carnitine palmitoyltransferase 1 (CPT1) inhibitor etomoxir (40 μM), agonist of M3 muscarinic acetylcholine receptor carbachol (100 μM) and PKC inhibitor chelerythrine (10 μM). Concentrations of the compounds were determined in optimization experiments. Compounds were obtained from Sigma Aldrich if not indicated.

2.7. Measurements of palmitate oxidation and glucose utilization

Palmitate oxidation and glucose utilization were determined by including during culture 2 μCi [^{3}H]palmitate and 2 μCi d-[5-^{3}H]glucose, respectively. Blanks for each condition were created by adding radioactive compounds to medium. After 1-h treatments, media were transferred to 1.5-ml tubes. Then tubes were placed inside scintillation vials containing 500 μl of H_2O. The scintillation vials were sealed and incubated at 56 °C overnight to permit ^3H_2O formed by the cells to evaporate and equilibrate with water in the vials [26]. The vials were then cooled to room temperature. After removing the tubes, 15 ml Ultima Gold™ scintillation fluid (PerkinElmer) was added to the water and ^3H_2O content determined by a liquid-scintillation spectrometer (Wallac System 1400™ PerkinElmer, Boston, MA). The average number of disintegrations in blank tubes was subtracted from experimental measurements.

2.8. Measurements of mitochondrial DNA

Total DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). The real-time PCR was performed using Dynamo Capillary SYBR Green qPCR kit (Finnzymes, Espoo, Finland). mtDNA was amplified using primers against ND1 gene (5′-ATTACTTCTGCCAGCCTGAC-3′

(forward) and 5′-GGGTCCTAGGAAGATAATAGTTG-3′ (reverse)). Nuclear DNA was amplified using primers against β-actin gene (5′-CCCTACAGTG CTGTGGGTTT-3′ (forward) and 5′-GAGACATGCAAGGAGTGCAA-3′ (reverse)). Changes in mtDNA number were calculated using the following formula: target amount $= 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [C_t\ (\text{ND1 treat}) - C_t\ (\beta\text{-actin treat})] - [C_t\ (\text{ND1 control}) - C_t\ (\beta\text{-actin control})$.

2.9. GSIS

After 48-h treatments, MIN6 cells were incubated for 30 min at 37 °C in the presence of 2 mM glucose in buffer containing 125 mM NaCl, 5.9 mM KCl, 1.2 mM $MgCl_2$, 1.3 mM $CaCl_2$ and 25 mM HEPES, titrated to pH 7.4 with NaOH, and supplemented

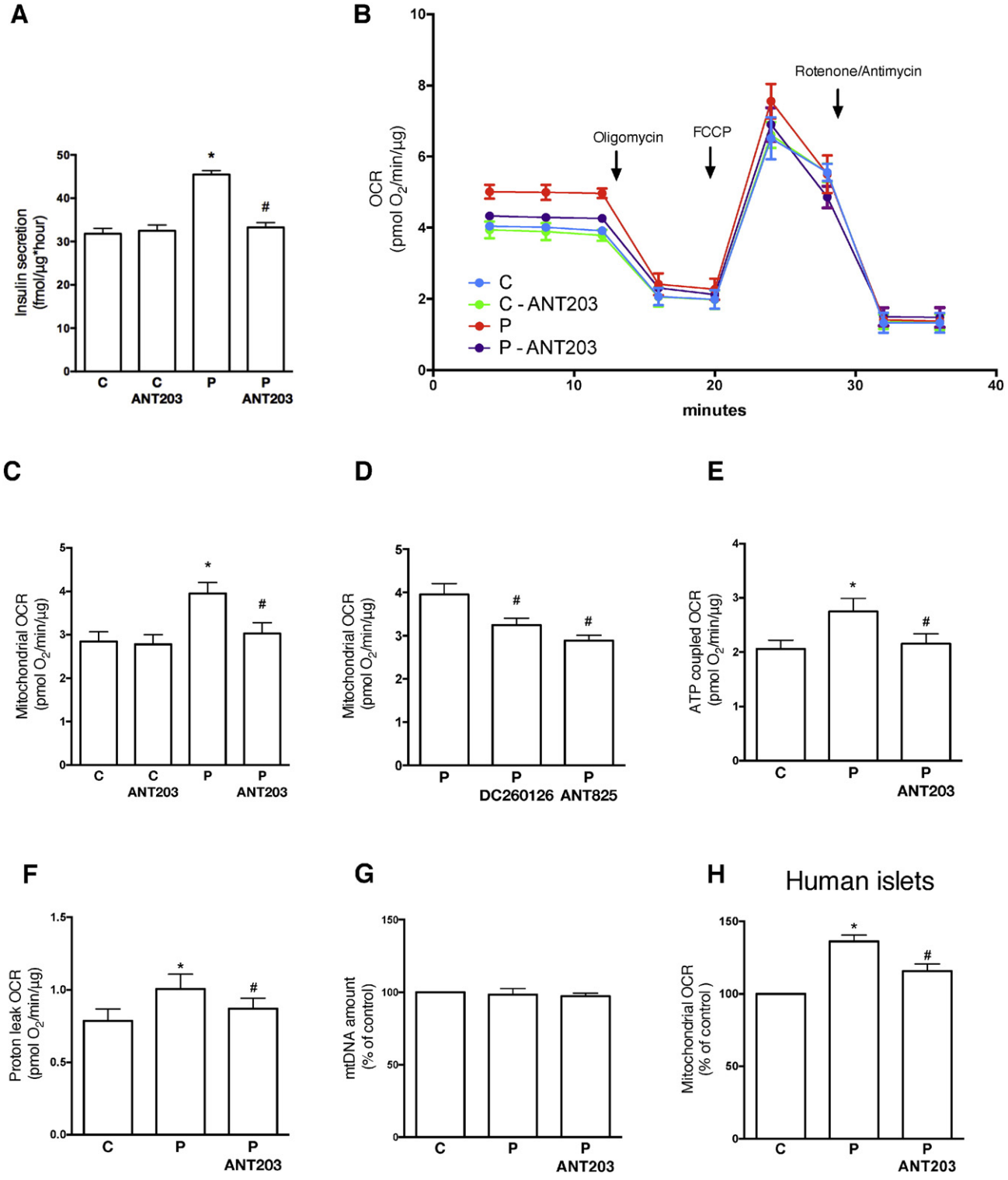
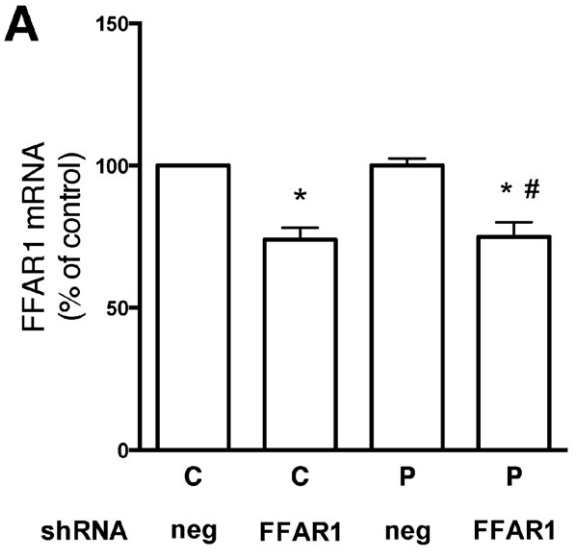
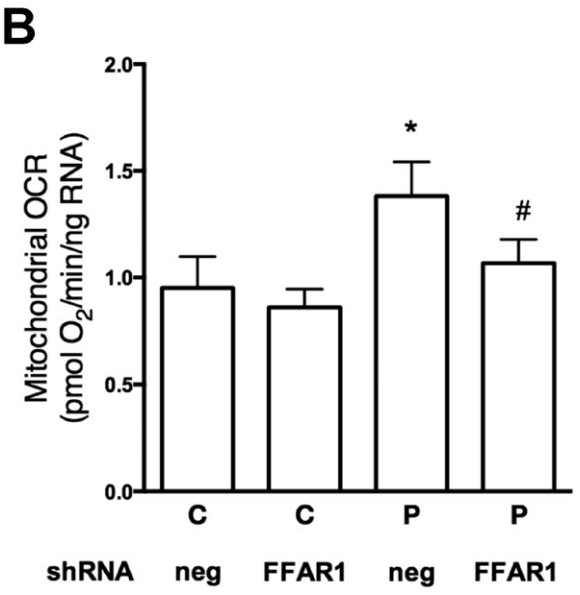
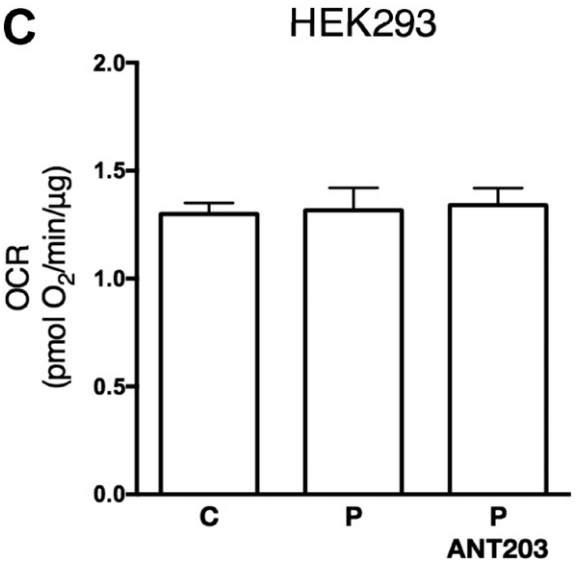


Fig. 1. Short-term palmitate exposure enhances mitochondrial respiration via FFAR1. MIN6 cells (A–G) or human islets (H) were cultured in XF assay medium in the absence (C) or presence of 0.5 mM palmitate (P), 2 μM ANT203, 2 μM ANT825 or 10 μM DC260126 for 1 h. A. Amount of insulin secreted during exposure. B. OCR from a representative experiment where each point is a mean ± SD of 5 replicates. 5 μM oligomycin, 4 μM FCCP and mixture of 5 μM of rotenone and 5 μM antimycin were added as indicated. C and D. Mitochondrial OCR. E. ATP-coupled OCR. F. Proton leak OCR. G. Mitochondrial DNA amount. H. Mitochondrial OCR in human islets. A, C–H. Data are mean ± SEM of 3–6 independent experiments. Statistical analysis: RM one-way ANOVA (with Bonferroni's post hoc test) (A, C–F), paired t-test (G and H). * vs control, # vs palmitate. $p < 0.05$ was considered statistically significant.







with 0.1% fatty acid free BSA (fraction V; Boehringer Mannheim GmbH, Germany). After this incubation period, the buffer was changed to the same type of buffer containing either 2 or 20 mM glucose. Cells were incubated for 30 min and then aliquots of the buffer were collected [17].

Insulin was determined by a competitive ELISA and normalized to intracellular protein content. To isolate protein, cells were washed with PBS and lysed in the same buffer with the addition of 1% Triton X100 and 0.4% protease inhibitor cocktail (both obtained from Sigma Aldrich). Protein content in the lysates was determined by the DC Protein Assay (BioRad, Hercules, CA, USA).

2.10. Statistical analysis

All analysis and figure presentation was done with Graph Pad Prism software, version 6 (San Diego, CA, USA). Paired t-test and repeated measures one-way ANOVA (with Bonferroni post hoc test and Fishers LSD test) were used for statistical analysis. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Short-term palmitate exposure enhances mitochondrial respiration via FFAR1

MIN6 cells cultured for 1 h in the presence of palmitate secreted 50% more insulin than in the absence of the fatty acid (Fig. 1A). This was accompanied by a 35% rise in mitochondrial OCR (Fig. 1B and C). Inhibition of the FFAR1 signaling pathway with the receptor antagonist ANT203 prevented the palmitate-induced rise in insulin secretion (Fig. 1A). The effect was associated with a significant reduction in OCR to levels close to those observed in control cells (Fig. 1B and C). In the absence of palmitate, ANT203 showed no effects on insulin secretion and OCR (Fig. 1A, B and C). Similar results were obtained when structurally different antagonists DC260126 [27] or ANT825 [24] were used in combination with palmitate (Fig. 1D). Palmitate- and ANT203-induced changes in OCR were due to proportionate changes in ATP-coupled respiration and proton leak (Fig. 1E and F). Mitochondrial DNA amount was not affected by treatments suggesting that the observed effects were due to changes in bioenergetics (Fig. 1G).

The role of FFAR1 in palmitate-induced elevation of OCR was verified in human islets. One-hour treatment of the islets with palmitate increased OCR by ~35% (Fig. 1H). When ANT203 was also present during culture, OCR was significantly lowered (Fig. 1H).

To further confirm the role of FFAR1 in the regulation of mitochondrial respiration, we knocked down the expression of the receptor by using shRNA technique. With this approach mRNA level of the receptor was reduced by 25–35% (Fig. 2A). When FFAR1-deficient MIN6 cells were treated with palmitate for 1 h, no statistically significant changes in OCR were observed (Fig. 2B).

Finally, to validate the role of FFAR1 in the regulation of mitochondrial respiration, the human cell line HEK293 that does not express the receptor [5] was cultured with or without palmitate and ANT203.

Fig. 2. Short-term palmitate exposure does not enhance mitochondrial respiration in FFAR1-deficient MIN6 and HEK293 cells. MIN6 cells (A and B) or HEK293 cells (C) were cultured in XF assay medium in the absence (C) or presence of 0.5 mM palmitate (P). A. The FFAR1 mRNA level was normalized to the actin mRNA level and presented as fold control in MIN6 cells transfected with FFAR1 shRNA or a negative control shRNA (neg). B. Mitochondrial OCR in the transfected MIN6 cells in the absence or presence of palmitate. C. Mitochondrial OCR in HEK293 cells. Data are mean ± SEM of 4 independent experiments. Statistical analysis: paired t-test (A), RM one-way ANOVA (with Bonferroni's post hoc test) (B and C). * vs control transfected with negative shRNA, # vs palmitate transfected with negative shRNA. $p < 0.05$ was considered statistically significant.

Treatment of these cells with palmitate in the absence or presence of ANT203 induced no changes in OCR (Fig. 2C).

Taken together our findings demonstrate that the palmitate-induced increase in mitochondrial respiration is dependent on FFAR1 signaling.

3.2. $G\alpha_q$ protein-coupled signaling enhances mitochondrial respiration in the presence of extracellular palmitate

To further validate the role of FFAR1 signaling, MIN6 cells were treated with FFAR1 agonists TUG469 or AS2034178. The agonists induced

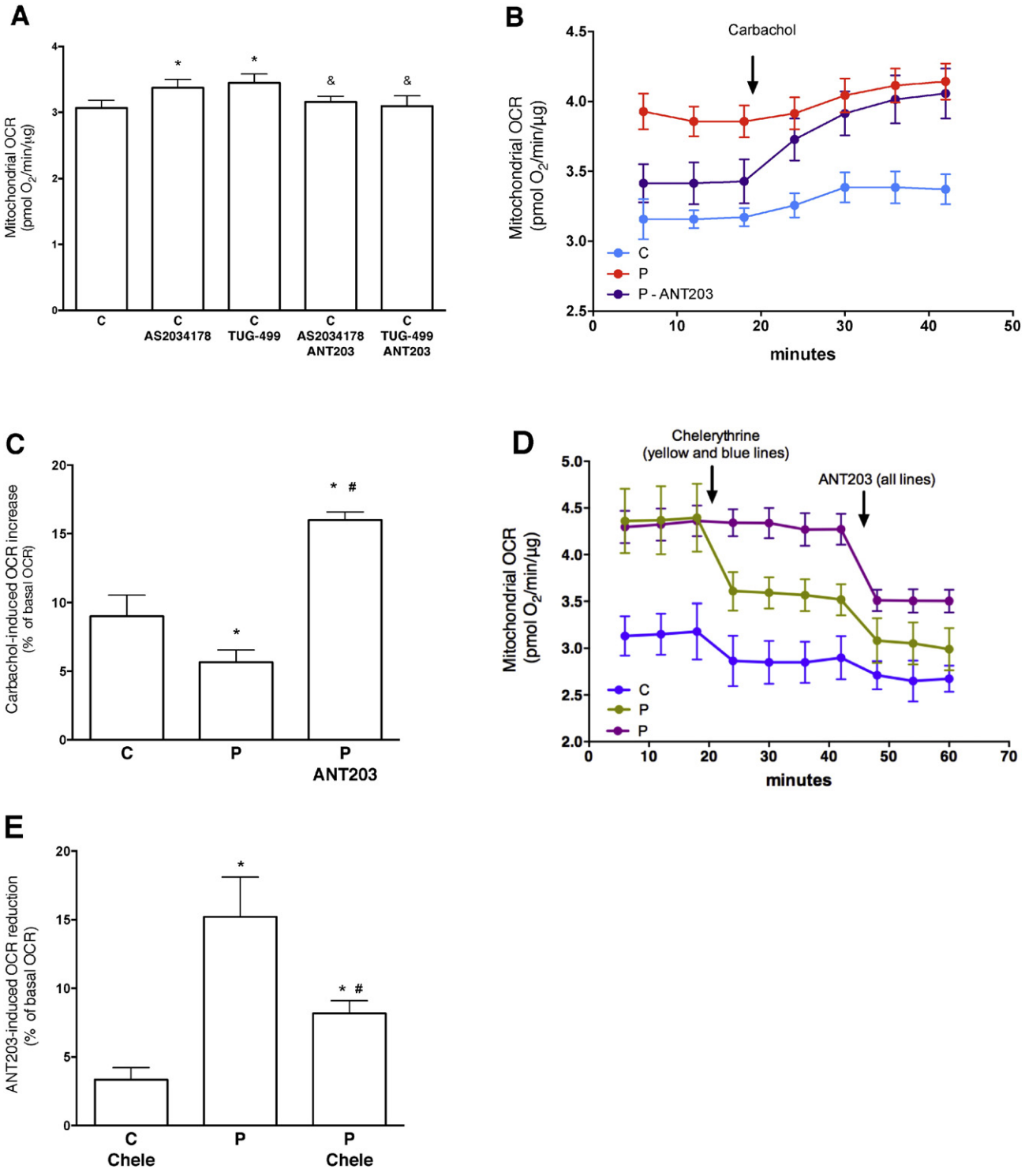


Fig. 3. $G\alpha_q$ protein-coupled signaling enhances mitochondrial respiration in the presence of extracellular palmitate. MIN6 cells were cultured in XF assay medium in the absence (C) or presence of 0.5 mM palmitate (P), 2 μM AS2034178, 2 μM TUG-499 or 10 μM ANT203 for 1 h. A. Effect of FFAR1 agonists on OCR. B and D. OCR from representative experiments where each point is a mean ± SD from 5 replicates. Carbachol (100 μM), chelerythrine (10 μM) and ANT203 (2 μM) were added as indicated. C. Effect of carbachol on mitochondrial OCR in palmitate-treated cells in the absence or presence of ANT203. E. Effect of ANT203 on mitochondrial OCR in palmitate-treated cells in the absence or presence of chelerythrine. A, C and E. Data are mean ± SEM of 3–6 independent experiments. Statistical analysis: RM one-way ANOVA (with Fisher's LSD test) (A), RM one-way ANOVA (with Bonferroni's post hoc test) (C–E). * vs control, # vs palmitate, & vs. corresponding condition without ANT203. $p < 0.05$ was considered statistically significant.

only 5–10% rise in OCR (Fig. 3A). These results indicate that palmitate enhances mitochondrial respiration via combined action of intracellular fatty acid metabolism and FFAR1 signaling.

Next, we explored the role of $G\alpha_q$-dependent signaling in the regulation of OCR. This was done by stimulating the $G\alpha_q$-coupled M3 muscarinic acetylcholine receptor. Addition of the receptor agonist, carbachol, to palmitate-treated cells caused a modest increase in OCR (Fig. 3B). However, when ANT203 was also present during culture, the increase in OCR was more pronounced (Fig. 3B). In the absence of extracellular palmitate, carbachol caused minor changes in OCR (Fig. 3B). Altogether, it suggests that signaling of other $G\alpha_q$ protein-coupled receptors may also interplay with metabolism and affect mitochondrial respiration of beta cells.

ER Ca^{2+} release and protein kinase C (PKC) are downstream mediators of $G\alpha_q$-dependent signaling [7]. The importance of Ca^{2+} for mitochondrial function is well established. It has been demonstrated that the activity of three key mitochondrial dehydrogenases and ATP synthase is sensitive to Ca^{2+} [28–30]. Moreover, IP_3-dependent Ca^{2+} release to mitochondria is required for efficient mitochondrial respiration of eukaryotic cells [31]. The importance of PKC for mitochondrial

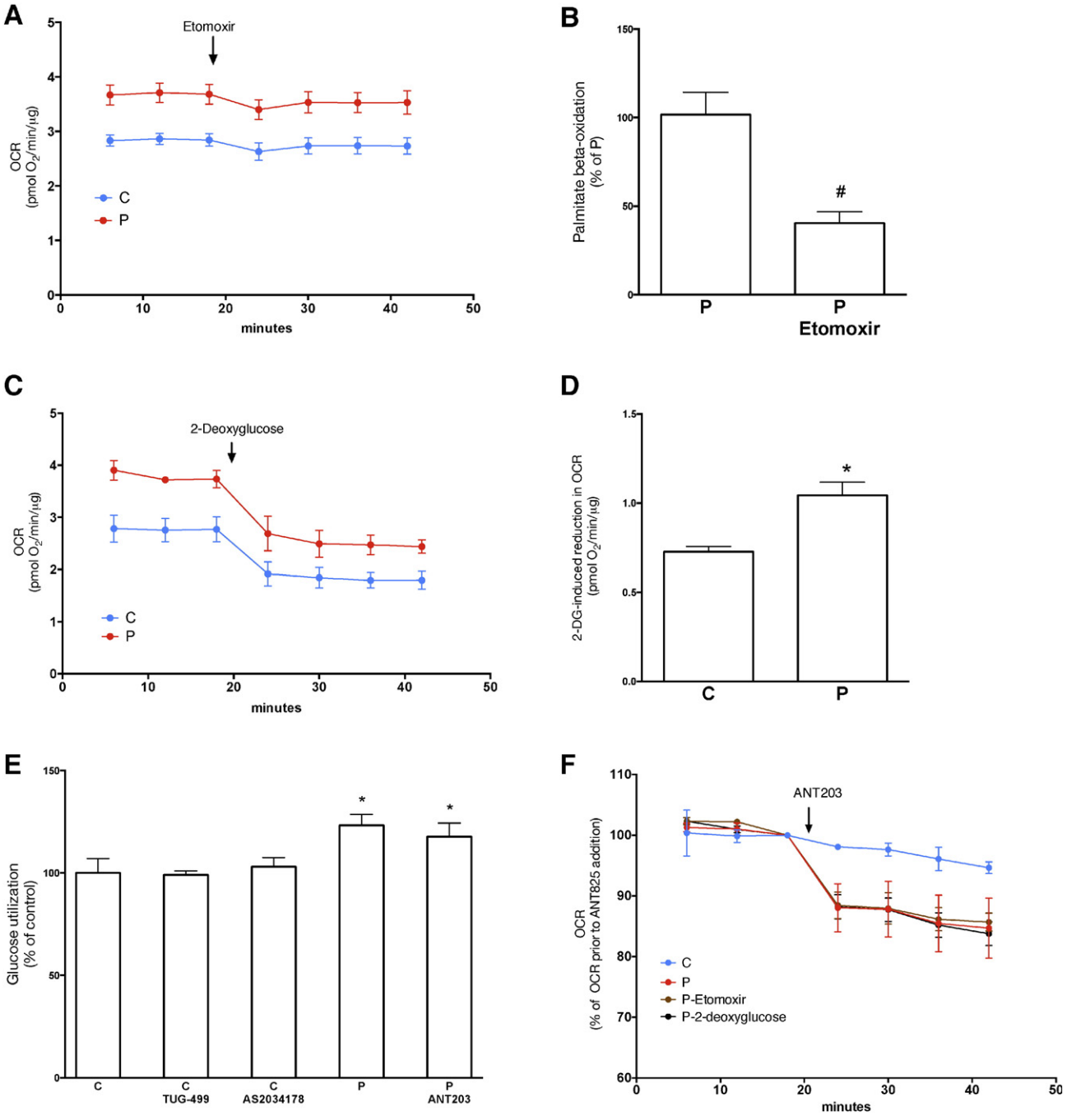


Fig. 4. Intracellular metabolism of palmitate enhances glucose utilization whereas FFAR1 signaling enhances mitochondrial function. MIN6 cells were cultured in XF assay medium in the absence (C) or presence of 0.5 mM palmitate (P), 40 μM etomoxir, 100 mM 2-deoxyglucose, 2 μM ANT203, 2 μM AS2034178 or 2 μM TUG-499 for 1 h. A, C, and F. During culture, 40 μM etomoxir, 100 mM 2-deoxyglucose and 2 μM ANT203 were added as indicated. OCR from representative experiments where each point is a mean ± SD from 5 replicates. B. Palmitate oxidation during 1-h treatment estimated by the formation of ^3H_2O from ^3H-labeled palmitate. D. Reduction in OCR after addition of 2-DG. E. Glucose utilization during 1-h treatment determined by the formation of ^3H_2O from d-[5-^{3}H]glucose. B, D, and E. Data are mean ± SEM of 3–4 independent experiments. Statistical analysis: paired t-test (B and D) and RM one-way ANOVA (with Bonferroni's post hoc test) (E). * vs control, # vs palmitate. $p < 0.05$ was considered statistically significant.

respiration has recently been demonstrated on HeLa and embryonic stem cells [32,33]. To further explore the role of $G\alpha_q$ protein-coupled signaling, we examined the involvement of PKC in FFAR1-mediated regulation of mitochondrial respiration. When PKC inhibitor chelerythrine was added to MIN6 cells, OCR was lowered. The decrease was more pronounced in palmitate-treated cells, however, suggesting that the palmitate-induced increase in OCR is PKC-dependent (Fig. 3D). When ANT203 was added to the cells, where chelerythrine was present, reduction of OCR was less than in the presence of palmitate alone (Fig. 3D and E) implying that FFAR1-mediated effects on mitochondrial respiration involve PKC.

3.3. Intracellular metabolism of palmitate enhances glucose utilization whereas FFAR1 signaling enhances mitochondrial function

We next asked if the observed rise in OCR during the palmitate exposure was due to elevated oxidation of the fatty acid. When β-oxidation was inhibited in palmitate-treated MIN6 cells by adding the CPT1 inhibitor etomoxir, OCR was lowered only transiently and modestly (Fig. 4A). At the same time, palmitate oxidation was effectively inhibited by more than 50% under these conditions as determined by using ^{3}H-palmitate (Fig. 4B). These results indicate that the contribution of β-oxidation to the increase in OCR is minor. When glycolysis was inhibited by the addition of the non-metabolisable glucose analog 2-DG, OCR was lowered in control and in palmitate-treated cells (Fig. 4C). The reduction was more accentuated in palmitate-treated cells, however. It suggests that the rise in OCR in palmitate-treated cells was largely due to increased glucose oxidation (Fig. 4C and D). In line with these results, in the presence of palmitate glucose utilization was increased compared to control cells (Fig. 4E). We asked if the FFAR1 signaling pathway elevates OCR by enhancing glucose utilization. Surprisingly, FFAR1 antagonist ANT203 did not reduce glucose utilization (Fig. 4E). Furthermore, FFAR1 agonists TUG-499 and AS2034178 in the absence of extracellular palmitate did not affect glucose utilization (Fig. 4E). These results suggest that FFAR1 signaling elevates OCR by enhancing mitochondrial function rather than glucose utilization. Indeed, when the antagonist was added to palmitate-treated MIN6 cells, OCR was rapidly reduced by 15–20% irrespective of the absence or presence of 2-DG or etomoxir (Fig. 4F).

Studies addressing the contribution of FFAR1 to the regulation of intracellular metabolism are controversial. Steneberg et al. showed that overexpression of FFAR1 in beta cells leads to perturbations in glucose and fatty acid metabolism [34]. On the other hand, Alquer et al. observed no changes in intracellular metabolism of islets isolated from FFAR1 knockout mice [35]. The discrepancy may be explained by the differences in the level of circulating free fatty acids. As it is evident from our study, FFAR1 signaling significantly affects intracellular metabolism in the presence of high palmitate. Further support to the involvement of FFAR1 in the regulation of metabolism provides a recent study that demonstrates that FFAR1 pathway affects glycerolipid formation [36].

3.4. FFAR1 signaling is involved in amplification of insulin secretion by etomoxir in the presence of extracellular palmitate

Increased generation of metabolites via GL/FFA cycle stimulates insulin secretion. To address the involvement of the FFAR1 pathway in GL/FFA cycle-induced stimulation of insulin secretion, we directed fatty acids towards accumulation by inhibiting β-oxidation with etomoxir. When etomoxir was present during culture with palmitate OCR was slightly elevated (Fig. 5A). As expected, palmitate-induced insulin secretion was further amplified in the presence of etomoxir (Fig. 5B). When ANT203 was also present during culture with palmitate and etomoxir, mitochondrial respiration and insulin secretion were reduced to control levels (Fig. 5A and B). These results suggest that FFAR1 signaling is required for amplification of insulin secretion by accumulated fatty acid species.

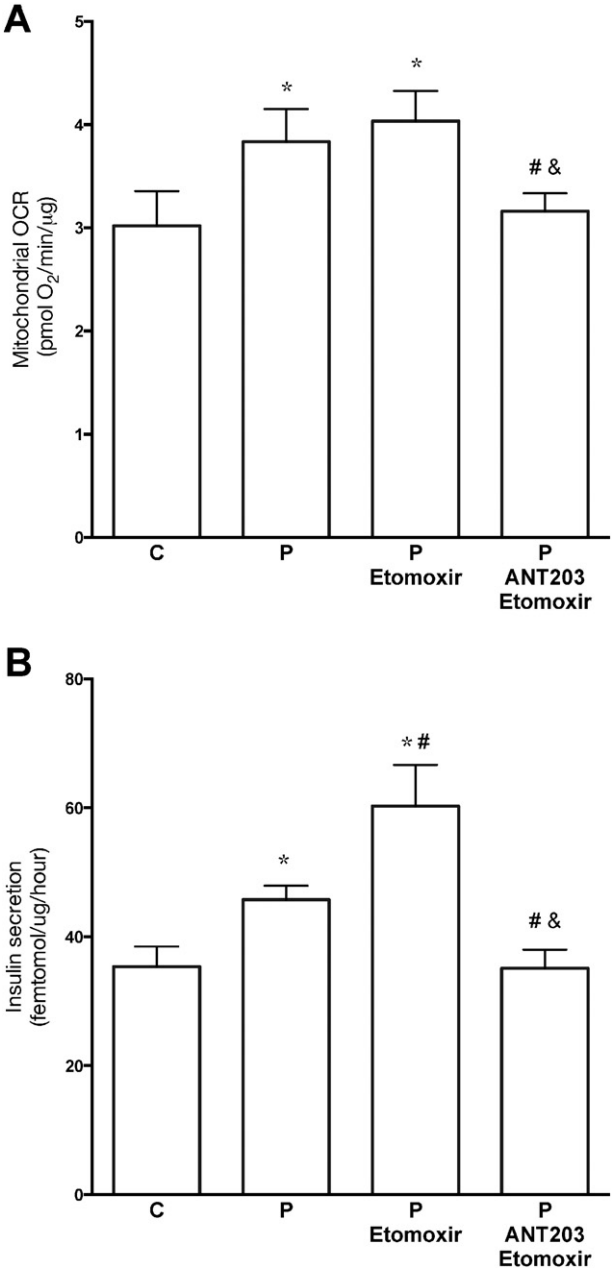


Fig. 5. FFAR1 signaling is involved in amplification of insulin secretion by etomoxir in the presence of extracellular palmitate. MIN6 cells were cultured in XF assay medium in the absence (C) or presence of 0.5 mM palmitate (P), 2 μM ANT203 or 40 μM etomoxir for 1 h. A. Mitochondrial OCR during the culture. B. Amount of insulin secreted during exposure. Data are mean ± SEM of 6 independent experiments. Statistical analysis: RM one-way ANOVA (with Bonferroni's post hoc test). * vs control, # vs palmitate, & vs palmitate + etomoxir. $p < 0.05$ was considered statistically significant.

Based on our findings we propose a model of fatty acid-induced stimulation of insulin secretion (Fig. 6). Intracellular metabolism of fatty acids enhances glucose utilization and elevates the level of glycerol-3-phosphate required for the formation of triacylglycerols. In parallel, beta cells sense extracellular fatty acids via FFAR1 and respond by enhancing mitochondrial function. It facilitates oxidation of metabolites from the already accelerated glycolytic pathway. Elevated generation of ATP initiates and maintains energy-demanding GL/FFA cycling [4] and, thereby, stimulates insulin secretion. In support of this

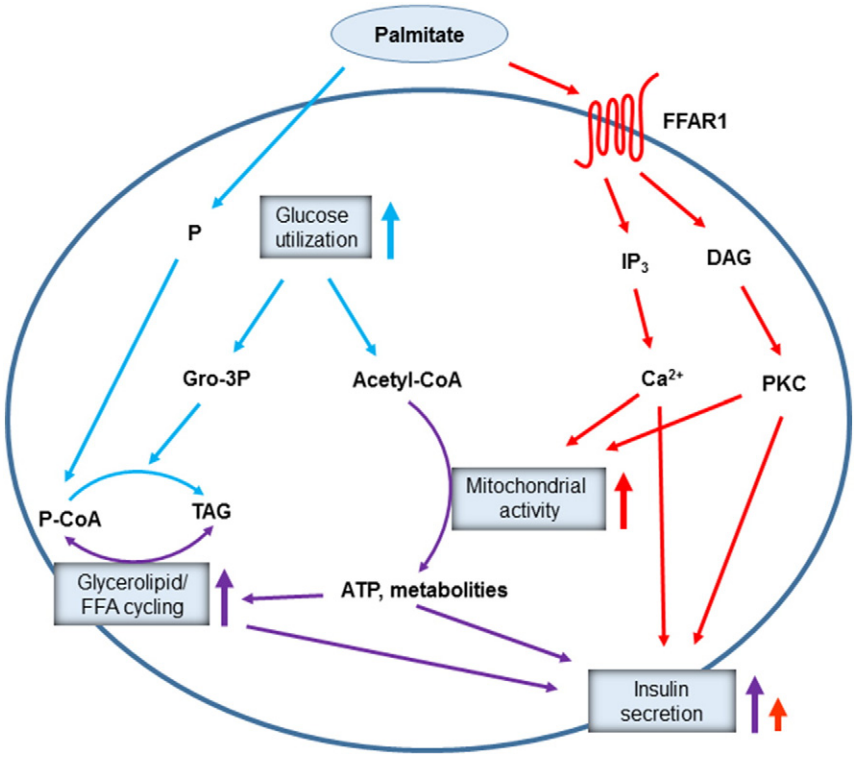


Fig. 6. A model of integrated action of intracellular fatty acid metabolism and FFAR1 signaling on mitochondrial activity and insulin secretion. At high palmitate (P), intracellular metabolism of the fatty acid enhances glucose utilization and elevates the level of glycerol-3-phosphate (Gro-3P) required for the formation of triacylglycerols (TAG). In parallel, beta cells sense extracellular fatty acids via FFAR1. Activation of the receptor leads to the activation of phospholipase C and hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG and IP_3 activate protein kinase C (PKC) and trigger ER Ca^{2+} release, respectively. It leads to enhanced mitochondrial function, which facilitates oxidation of glucose metabolites from the already accelerated glycolytic pathway. Elevated generation of ATP initiates and maintains glycerolipid/FFA cycle and, thereby, stimulates insulin secretion. In parallel, increased PKC activity and increased ER Ca^{2+} release directly stimulate insulin secretion, which constitutes a small portion of total secreted insulin. Blue lines—action of intracellular metabolism of fatty acids. Red lines—action of FFAR1 signaling. Purple lines—actions dependent on two pathways.

hypothesis, a recent study showed that in palmitate-treated INS-1 832/3 cells GL/FFA cycle is inhibited in the presence of FFAR1 antagonist [36].

The proposed model does not exclude mitochondria-independent action of FFAR1 signaling on insulin secretion. We and others have demonstrated that a FFAR1 agonist potentiates insulin secretion in the absence of extracellular fatty acid, although to a much lesser extent [17,37].

3.5. Presence of FFAR1 antagonist during long-term palmitate exposure resulted in elevated mitochondrial respiration and preserved insulin secretion

After 48-h exposure to palmitate, glucose-stimulated insulin secretion (GSIS) from MIN6 cells was attenuated to 40% of that in control cells (Fig. 7A). Also, insulin content was reduced by 65% (Fig. 7B). Surprisingly, OCR in palmitate-treated cells was almost similar to OCR in non-treated cells (Fig. 7C and D). However, ATP-coupled OCR was slightly reduced whereas proton leak OCR was slightly elevated (Fig. 7E and F). As a result, ATP-coupling efficiency (the ratio between ATP-coupled and proton leak OCR) was reduced from 2.1 in control cells to 1.5 in palmitate-treated cells (Fig. 7G). When ANT203 was also present during palmitate exposure, GSIS and insulin content were preserved (Fig. 7A and B). At the same time, mitochondrial OCR was increased to levels significantly higher than in control cells (Fig. 7C and D). Similar elevation was observed when two other structurally dissimilar antagonists, DC260126 and ANT825, were used (Fig. 7D). However, ATP-coupling efficiency was not improved (Fig. 7E, F and G). Amount of mitochondrial DNA was not affected by culture conditions indicating that the observed drastic effect of the antagonist on OCR was not accounted for by a change in mitochondrial number (Fig. 7H).

The role of FFAR1 in the regulation of mitochondrial respiration during the long-term exposure to palmitate was further addressed by shRNA-mediated silencing of the receptor. We found that after 48-h treatment with palmitate OCR was highly elevated in FFAR1-silenced cells but not in cells transfected with control vector encoding non-mammalian shRNA (Fig. 7I).

Increased proton leak may, at least partially, explain the impaired GSIS after long-term culture with palmitate. In beta cells, uncoupling plays an important role in the control of ATP/ADP ratio [21]. It has been demonstrated that increased levels of mitochondria carrier protein, uncoupling protein 2 (UCP2), alter GSIS [38,39]. Mechanisms by which fatty acids uncouple respiration include cycling between the protonated and the unprotonated forms [40] and changes in mitochondrial fission-fusion events [41]. The role of uncoupling in beta cells is controversial, however. Although it is often regarded as being detrimental, it also has a cytoprotective role due to diminished reactive oxygen species (ROS) production [42–44]. However, the FFAR1 antagonist was unable to improve ATP-coupling efficiency in our study suggesting that intracellular action of fatty acid plays the major role in the uncoupling of mitochondria. Instead, by attenuating respiration and lowering insulin secretion during culture, the antagonist preserved fuel and insulin content and thereby enhanced mitochondrial respiration and improved insulin secretion after culture. Studies have demonstrated that inhibition of FFAR1 during long-term palmitate exposure lowers generation of ROS and alleviates apoptosis [17,18,20,45,46]. Interestingly, in one study FFAR1 agonist TUG-469 was also protective during culture with palmitate. We assume that it might be due to partial agonistic properties of the compound that could make it act as an antagonist when combined with such a strong agonist as palmitate [47]. Taking into account the major contribution of mitochondrial electron transport chain to the

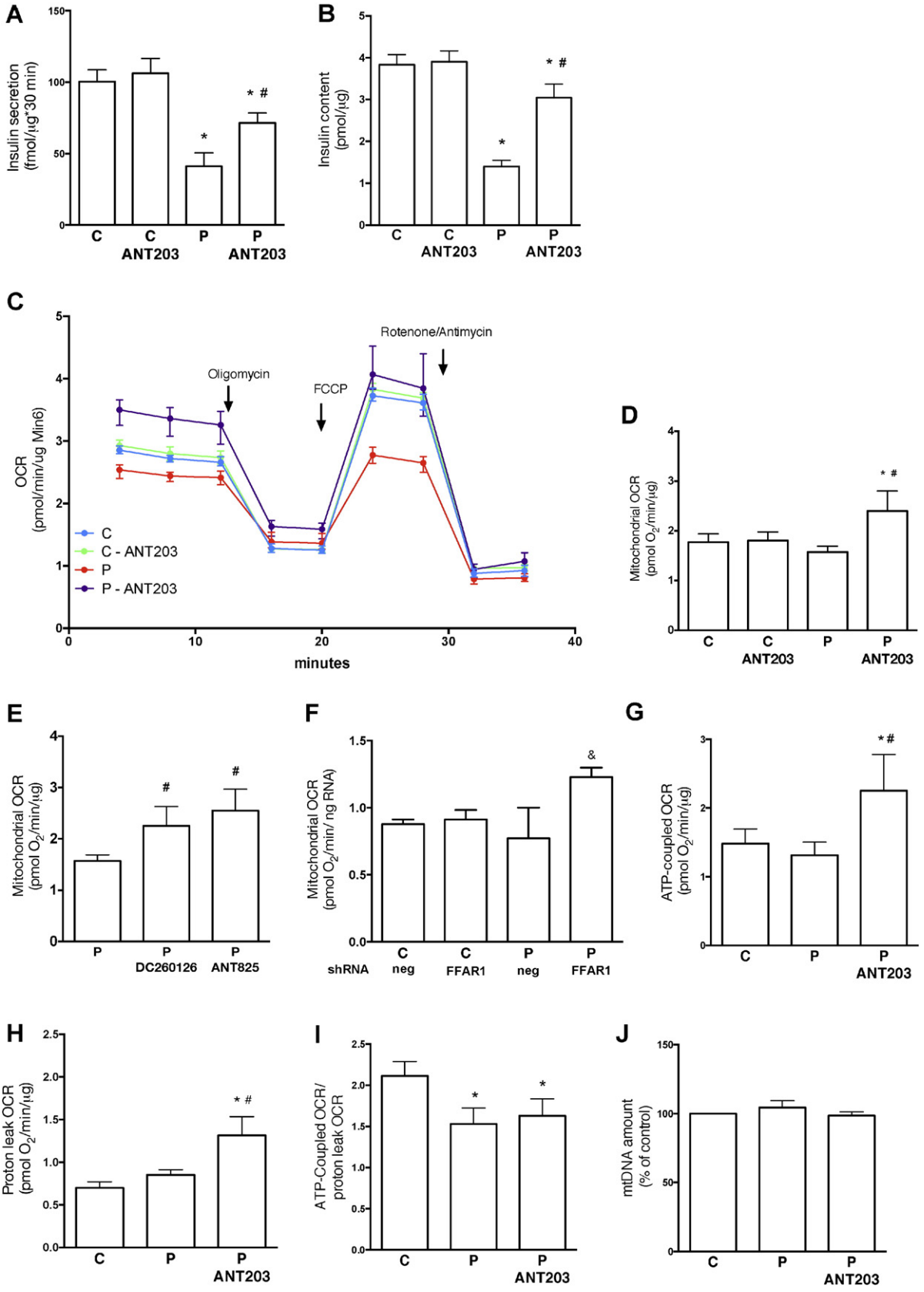


Fig. 7. Presence of FFAR1 antagonist during long-term palmitate exposure resulted in elevated mitochondrial respiration and preserved insulin secretion. MIN6 cells were cultured in the absence (C) or presence of 0.5 mM palmitate (P), with or without 2 µM ANT203, 2 µM ANT825 or 10 µM DC260126 for 48 h. After culture, GSIS, insulin content, OCR and mitochondrial DNA amount were determined. A. GSIS during 30 min. B. Insulin content. C. OCR from a representative experiment where each point is a mean ± SD of 5 replicates. 5 µM oligomycin, 4 µM FCCP and mixture of 5 µM of rotenone and 5 µM antimycin were added as indicated. D and E. Mitochondrial OCR. F. Mitochondrial OCR in cells transfected with either FFAR1 shRNA or negative control shRNA. G. ATP-coupled OCR. H. Proton leak OCR. I. ATP-coupling efficiency (ratio between ATP-coupled and proton leak OCR). J. Mitochondrial DNA amount. A, B, D–J. Data are mean ± SEM of 3–5 independent experiments. Statistical analysis: RM one-way ANOVA (with Bonferroni's post hoc test) (A–I), paired t-test (J). * vs control, # vs palmitate, & vs palmitate transfected with negative shRNA. $p < 0.05$ was considered statistically significant.

generation of ROS [48], one may speculate that, by reducing mitochondrial respiration during culture, a FFAR1 antagonist reduces ROS generation and in such a way, lowers apoptosis.

4. Conclusions

In summary, during palmitate exposure, integrated action of intracellular metabolism of the fatty acid and $G\alpha_q$-coupled FFAR1 signaling on mitochondrial respiration underlies the synergistic action of the two pathways on insulin secretion.

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References

[1] C. Berne, The metabolism of lipids in mouse pancreatic islets. The biosynthesis of triacylglycerols and phospholipids, Biochem. J. 152 (3) (1975) 667–673.
[2] M. Prentki, S. Vischer, M.C. Glennon, R. Regazzi, J.T. Deeney, B.E. Corkey, Malonyl-CoA and long chain acyl-CoA esters as metabolic coupling factors in nutrient-induced insulin secretion, J. Biol. Chem. 267 (9) (1992) 5802–5810.
[3] E. Sargsyan, P. Bergsten, Lipotoxicity is glucose-dependent in INS-1E cells but not in human islets and MIN6 cells, Lipids Health Dis. 10 (2011) 115.
[4] M. Prentki, S.R. Madiraju, Glycerolipid metabolism and signaling in health and disease, Endocr. Rev. 29 (6) (2008) 647–676.
[5] C.P. Briscoe, M. Tadayyon, J.L. Andrews, W.G. Benson, J.K. Chambers, M.M. Eilert, et al., The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids, J. Biol. Chem. 278 (13) (2003) 11303–11311.
[6] Y. Itoh, Y. Kawamata, M. Harada, M. Kobayashi, R. Fujii, S. Fukusumi, et al., Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40, Nature 422 (6928) (2003) 173–176.
[7] K. Fujiwara, F. Maekawa, T. Yada, Oleic acid interacts with GPR40 to induce $Ca2^+$ signaling in rat islet beta-cells: mediation by PLC and L-type $Ca2^+$ channel and link to insulin release, Am. J. Physiol. Endocrinol. Metab. 289 (4) (2005) E670–E677.
[8] H. Shapiro, S. Shachar, I. Sekler, M. Hershfinkel, M.D. Walker, Role of GPR40 in fatty acid action on the beta cell line INS-1E, Biochem. Biophys. Res. Commun. 335 (1) (2005) 97–104.
[9] T. Araki, M. Hirayama, S. Hiroi, K. Kaku, GPR40-induced insulin secretion by the novel agonist TAK-875: first clinical findings in patients with type 2 diabetes, Diabetes Obes. Metab. 14 (3) (2012) 271–278.
[10] C.F. Burant, P. Viswanathan, J. Marcinak, C. Cao, M. Vakilynejad, B. Xie, et al., TAK-875 versus placebo or glimepiride in type 2 diabetes mellitus: a phase 2, randomised, double-blind, placebo-controlled trial, Lancet 379 (9824) (2012) 1403–1411.
[11] E. Leifke, H. Naik, J. Wu, P. Viswanathan, D. Demanno, M. Kipnes, et al., A multiple-ascending-dose study to evaluate safety, pharmacokinetics, and pharmacodynamics of a novel GPR40 agonist, TAK-875, in subjects with type 2 diabetes, Clin. Pharmacol. Ther. 92 (1) (2012) 29–39.
[12] H. Naik, M. Vakilynejad, J. Wu, P. Viswanathan, N. Dote, T. Higuchi, et al., Safety, tolerability, pharmacokinetics, and pharmacodynamic properties of the GPR40 agonist TAK-875: results from a double-blind, placebo-controlled single oral dose rising study in healthy volunteers, J. Clin. Pharmacol. 52 (7) (2012) 1007–1016.
[13] Y.P. Zhou, V.E. Grill, Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle, J. Clin. Invest. 93 (2) (1994) 870–876.
[14] K. Iizuka, H. Nakajima, M. Namba, J. Miyagawa, J. Miyazaki, T. Hanafusa, et al., Metabolic consequence of long-term exposure of pancreatic beta cells to free fatty acid with special reference to glucose insensitivity, Biochim. Biophys. Acta 1586 (1) (2002) 23–31.
[15] E. Sargsyan, H. Ortsater, K. Thorn, P. Bergsten, Diazoxide-induced beta-cell rest reduces endoplasmic reticulum stress in lipotoxic beta-cells, J. Endocrinol. 199 (1) (2008) 41–50.
[16] L. Manukyan, S.J. Ubhayasekera, J. Bergquist, E. Sargsyan, P. Bergsten, Palmitate-induced impairments of beta-cell function are linked with generation of specific ceramide species via acylation of sphingosine, Endocrinology (2014), en20141467.
[17] H. Kristinsson, D.M. Smith, P. Bergsten, E. Sargsyan, FFAR1 is involved in both the acute and chronic effects of palmitate on insulin secretion, Endocrinology 154 (11) (2013) 4078–4088.
[18] A. Natalicchio, R. Labarbuta, F. Tortosa, G. Biondi, N. Marrano, A. Peschechera, et al., Exendin-4 protects pancreatic beta cells from palmitate-induced apoptosis by interfering with GPR40 and the MKK4/7 stress kinase signalling pathway, Diabetologia 56 (11) (2013) 2456–2466.
[19] P. Sun, T. Wang, Y. Zhou, H. Liu, H. Jiang, W. Zhu, et al., DC260126: a small-molecule antagonist of GPR40 that protects against pancreatic beta-cells dysfunction in db/db mice, PLoS One 8 (6) (2013), e66744.
[20] J. Wu, P. Sun, X. Zhang, H. Liu, H. Jiang, W. Zhu, et al., Inhibition of GPR40 protects MIN6 beta cells from palmitate-induced ER stress and apoptosis, J. Cell. Biochem. 113 (4) (2012) 1152–1158.
[21] C. Affourtit, M.D. Brand, Stronger control of ATP/ADP by proton leak in pancreatic beta-cells than skeletal muscle mitochondria, Biochem. J. 393 (Pt 1) (2006) 151–159.
[22] E.D. Kennedy, P. Maechler, C.B. Wollheim, Effects of depletion of mitochondrial DNA in metabolism secretion coupling in INS-1 cells, Diabetes 47 (3) (1998) 374–380.
[23] A. Soejima, K. Inoue, D. Takai, M. Kaneko, H. Ishihara, Y. Oka, et al., Mitochondrial DNA is required for regulation of glucose-stimulated insulin secretion in a mouse pancreatic beta cell line, MIN6, J. Biol. Chem. 271 (42) (1996) 26194–26199.
[24] M.J. Waring, D.J. Baker, S.N.L. Bennett, A.G. Dossetter, M.F.R. Garcia, J. Georgsson, et al., Discovery of a series of 2-(pyridinyl)pyrimidines as potent antagonists of GPR40, Med. Chem. Commun. 6 (6) (2015) 1024–1029.
[25] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method, Methods 25 (4) (2001) 402–408.
[26] S. Malmgren, D.G. Nicholls, J. Taneera, K. Bacos, T. Koeck, A. Tamaddon, et al., Tight coupling between glucose and mitochondrial metabolism in clonal beta-cells is required for robust insulin secretion, J. Biol. Chem. 284 (47) (2009) 32395–32404.
[27] H. Hu, L.Y. He, Z. Gong, N. Li, Y.N. Lu, Q.W. Zhai, et al., A novel class of antagonists for the FFAs receptor GPR40, Biochem. Biophys. Res. Commun. 390 (3) (2009) 557–563.
[28] M. Boerries, P. Most, J.R. Gledhill, J.E. Walker, H.A. Katus, W.J. Koch, et al., $Ca2^+$-dependent interaction of S100A1 with F1-ATPase leads to an increased ATP content in cardiomyocytes, Mol. Cell. Biol. 27 (12) (2007) 4365–4373.
[29] R.M. Denton, P.J. Randle, B.R. Martin, Stimulation by calcium ions of pyruvate dehydrogenase phosphate phosphatase, Biochem. J. 128 (1) (1972) 161–163.
[30] R.M. Denton, D.A. Richards, J.G. Chin, Calcium ions and the regulation of NAD^+-linked isocitrate dehydrogenase from the mitochondria of rat heart and other tissues, Biochem. J. 176 (3) (1978) 899–906.
[31] C. Cardenas, R.A. Miller, I. Smith, T. Bui, J. Molgo, M. Muller, et al., Essential regulation of cell bioenergetics by constitutive InsP3 receptor $Ca2^+$ transfer to mitochondria, Cell 142 (2) (2010) 270–283.
[32] B. Mahato, P. Home, G. Rajendran, A. Paul, B. Saha, A. Ganguly, et al., Regulation of mitochondrial function and cellular energy metabolism by protein kinase C-lambda/iota: a novel mode of balancing pluripotency, Stem Cells 32 (11) (2014) 2880–2892.
[33] K. Sugawara, M. Fujikawa, M. Yoshida, Screening of protein kinase inhibitors and knockdown experiments identified four kinases that affect mitochondrial ATP synthesis activity, FEBS Lett. 587 (23) (2013) 3843–3847.
[34] P. Steneberg, N. Rubins, R. Bartoov-Shifman, M.D. Walker, H. Edlund, The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse, Cell Metab. 1 (4) (2005) 245–258.
[35] T. Alquier, M.L. Peyot, M.G. Latour, M. Kebede, C.M. Sorensen, S. Gesta, et al., Deletion of GPR40 impairs glucose-induced insulin secretion in vivo in mice without affecting intracellular fuel metabolism in islets, Diabetes 58 (11) (2009) 2607–2615.
[36] M. El-Azzouny, C.R. Evans, M.K. Treutelaar, R.T. Kennedy, C.F. Burant, Increased glucose metabolism and glycerolipid formation by fatty acids and GPR40 receptor signaling underlies the fatty acid potentiation of insulin secretion, J. Biol. Chem. 289 (19) (2014) 13575–13588.
[37] H. Yashiro, Y. Tsujihata, K. Takeuchi, M. Hazama, P.R. Johnson, P. Rorsman, The effects of TAK-875, a selective G protein-coupled receptor 40/free fatty acid 1 agonist, on insulin and glucagon secretion in isolated rat and human islets, J. Pharmacol. Exp. Ther. 340 (2) (2012) 483–489.
[38] C.B. Chan, D. De Leo, J.W. Joseph, T.S. McQuaid, X.F. Ha, F. Xu, et al., Increased uncoupling protein-2 levels in beta-cells are associated with impaired glucose-stimulated insulin secretion: mechanism of action, Diabetes 50 (6) (2001) 1302–1310.
[39] C. Fleury, M. Neverova, S. Collins, S. Raimbault, O. Champigny, C. Levi-Meyrueis, et al., Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia, Nat. Genet. 15 (3) (1997) 269–272.
[40] J. Gutknecht, Proton conductance caused by long-chain fatty acids in phospholipid bilayer membranes, J. Membr. Biol. 106 (1) (1988) 83–93.
[41] A.J. Molina, J.D. Wikstrom, L. Stiles, G. Las, H. Mohamed, A. Elorza, et al., Mitochondrial networking protects beta-cells from nutrient-induced apoptosis, Diabetes 58 (10) (2009) 2303–2315.
[42] S.S. Korshunov, V.P. Skulachev, A.A. Starkov, High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria, FEBS Lett. 416 (1) (1997) 15–18.
[43] G. Mattiasson, M. Shamloo, G. Gido, K. Mathi, G. Tomasevic, S. Yi, et al., Uncoupling protein-2 prevents neuronal death and diminishes brain dysfunction after stroke and brain trauma, Nat. Med. 9 (8) (2003) 1062–1068.
[44] Y. Teshima, M. Akao, S.P. Jones, E. Marban, Uncoupling protein-2 overexpression inhibits mitochondrial death pathway in cardiomyocytes, Circ. Res. 93 (3) (2003) 192–200.
[45] M.F. Graciano, M.M. Valle, R. Curi, A.R. Carpinelli, Evidence for the involvement of GPR40 and NADPH oxidase in palmitic acid-induced superoxide production and insulin secretion, Islets 5 (4) (2013) 139–148.
[46] S. Meidute Abaraviciene, I. Lundquist, J. Galvanovskis, E. Flodgren, B. Olde, A. Salehi, Palmitate-induced beta-cell dysfunction is associated with excessive NO production and is reversed by thiazolidinedione-mediated inhibition of GPR40 transduction mechanisms, PLoS One 3 (5) (2008), e2182.
[47] R. Wagner, G. Kaiser, F. Gerst, E. Christiansen, M.E. Due-Hansen, M. Grundmann, et al., Reevaluation of fatty acid receptor 1 as a drug target for the stimulation of insulin secretion in humans, Diabetes 62 (6) (2013) 2106–2111.
[48] A.J. Kowaltowski, N.C. de Souza-Pinto, R.F. Castilho, A.E. Vercesi, Mitochondria and reactive oxygen species, Free Radic. Biol. Med. 47 (4) (2009) 333–343.